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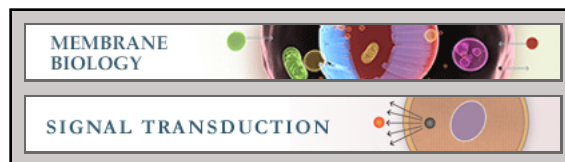
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Membrane Biology:
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An Electrostatic Switch Controls Palmitoylation of the Large Conductance Voltage- and Calcium-activated Potassium (BK) Channel*

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Background: Palmitoylation controls ion channel properties and function, but mechanisms that control palmitoylation are poorly defined.

Results: Phosphorylation of a polybasic domain upstream of palmitoylated cysteines controls BK channel palmitoylation and properties.

Conclusion: The polybasic domain is an electrostatic switch controlling palmitoylation.

Significance: Knowledge of how palmitoylation is regulated is essential for understanding the physiological role of this signaling mechanism in health and disease.

Protein palmitoylation is a major dynamic posttranslational regulator of protein function. However, mechanisms that control palmitoylation are poorly understood. In many proteins, palmitoylation occurs at cysteine residues juxtaposed to membrane-anchoring domains such as transmembrane helices, sites of irreversible lipid modification, or hydrophobic and/or polybasic domains. In particular, polybasic domains represent an attractive mechanism to dynamically control protein palmitoylation, as the function of these domains can be dramatically influenced by protein phosphorylation. Here we demonstrate that a polybasic domain immediately upstream of palmitoylated cysteine residues within an alternatively spliced insert in the C terminus of the large conductance calcium- and voltage-activated potassium channel is an important determinant of channel palmitoylation and function. Mutation of basic amino acids to acidic residues within the polybasic domain results in inhibition of channel palmitoylation and a significant right-shift in channel half maximal voltage for activation. Importantly, protein kinase A-dependent phosphorylation of a single serine residue within the core of the polybasic domain, which results in channel inhibition, also reduces channel palmitoylation. These data demonstrate the key role of the polybasic domain in controlling stress-regulated exon palmitoylation and suggests that phosphorylation controls the domain by acting as an electrostatic switch.

Protein S-palmitoylation is emerging as a major posttranslational modification to control a wide diversity of proteins, including ion channels (1, 2). Protein S-palmitoylation results

in the covalent linkage of the Cys-16 fatty acid palmitate to a cysteine residue via a thioester bond in a target protein. The reversible addition of palmitate may increase protein hydrophobicity and, therefore, can facilitate association of protein domains with the plasma membrane, regulation of signaling, and control of protein trafficking (1, 3–6). Sites of palmitoylation appear to have no canonical consensus sequence that can readily identify target cysteine residues within a protein. However, it is thought that additional membrane-targeting domains are required to be closely associated with sites of palmitoylation to allow efficient palmitoylation by the membrane-associated acyl palmitoyltransferases (zDHHCs)³ (1, 7). Indeed, in many proteins, palmitoylated cysteines are commonly found juxtaposed to transmembrane domains or at intracellular domains located close to additional membrane-targeting sequences such as other irreversible lipid anchors, hydrophobic regions, and polybasic domains (2, 4, 8, 9).

The role of polybasic domains adjacent to sites of palmitoylation is particularly intriguing as a potential mechanism to dynamically control palmitoylation of a target protein. For example, clusters of basic residues in proteins can function independently as membrane-targeting signals (10), and in several proteins, basic residues adjacent to palmitoylated cysteine residues have been shown to be important for efficient palmitoylation, most likely by controlling cysteine reactivity (11–13). Importantly, the electrostatic interactions of polybasic domains (either with phospholipids in the intracellular leaflet of the plasma membrane or acidic domains of proteins) may provide a dynamic mechanism for control of palmitoylation through reversible modification of local charge by other posttranslational modifications, such as protein phosphorylation. Thus,

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³ The abbreviations used are: zDHHC, acyl protein palmitoyl transferase gene family; BK, large conductance calcium- and voltage- activated potassium channel; STREX, stress-regulated exon; BH, basic hydrophobic; CSS, clustering and scoring strategy; PKA, cAMP-dependent protein kinase; RCK, regulator of potassium conductance; V0.5_{MAX}, voltage for half maximal conductance.

polybasic domains of proteins may represent an important, reversible role in the regulation of protein palmitoylation.

We recently identified that the intracellular C terminus of the large conductance voltage- and calcium-activated (BK) channel is palmitoylated within the alternatively spliced stress-regulated exon (STREX) (14). This C-terminal site of palmitoylation (cysteine residues C645:C646, Fig. 1) is not located close to a transmembrane domain, unlike the constitutively expressed palmitoylation site located in the S0-S1 linker (15). However, immediately upstream of the palmitoylated cysteines, a series of basic residues form a putative polybasic domain (Fig. 1) that may act as a potential additional membrane-targeting signal. Moreover, a serine residue (Ser-636) located at the midpoint of the polybasic domain has been shown previously to be a target for protein kinase A-mediated phosphorylation. Phosphorylation of Ser-636 results in channel inhibition (16, 17) and dissociation of the STREX domain from the plasma membrane (14).

In this work, we demonstrate that the polybasic region immediately upstream of the palmitoylated cysteine residues in the BK channel C terminus is an important determinant of BK channel palmitoylation. Mutation of basic residues to acidic residues results in a significant reduction in STREX palmitoylation, a significant right-shift in channel voltage for half-maximal activation, as well as reducing cell surface expression. Importantly, phosphorylation of Ser-636 acts as an electrostatic switch to control BK channel palmitoylation through the polybasic domain.

EXPERIMENTAL PROCEDURES

Channel Constructs and Expression—Subcloning and site-directed mutagenesis was performed on the murine BK channel α subunit as described previously (17, 18). Full-length channel constructs are numbered on the basis of the murine STREX variant of the BK channel (accession number AF156674) beginning at start methionine (MDALI...). Thus, the previously identified conserved palmitoylated cysteine residues at the 12th and 13th amino acid positions in the STREX insert and the upstream phosphorylation site (serine at the third amino acid position in the STREX insert) (14) are defined as C645:C646 and Ser-636, respectively, in this manuscript. Expression and analysis of C-terminal S6:COOH-GFP fusion constructs incorporating the entire C terminus, beginning at Gly-328 in pcDNA3.1, was performed as described previously (14). Full-length channels with an extracellular N-terminal FLAG tag and intracellular C-terminal HA tag constructs were created in pcDNA3.1 as described (15, 18, 19).

HEK293 Cell Culture and Immunofluorescence—HEK293 cells were maintained and transfected as described (17, 18) and transiently transfected by using Lipofectamine 2000 (Invitrogen). Extracellular FLAG tag epitopes on the BK channel were labeled in non-permeabilized HEK293 cells using mouse monoclonal anti-FLAG M2 antibody (Sigma) and Alexa Fluor 546-conjugated anti-mouse rabbit IgG (Molecular Probes). After fixing in 4% paraformaldehyde and quenching in 50 mM NH_4Cl , cells were permeabilized, and the intracellular C-terminal HA epitope tag was detected using anti-HA polyclonal rabbit antibody (Zymed Laboratories, Inc. and Alexa Fluor 647-

conjugated anti-rabbit IgG (Molecular Probes). Confocal images were acquired on a Zeiss LSM510 laser scanning microscope using a $\times 63$ oil Plan Apochromat (NA = 1.4) objective lens in multi-tracking mode. Quantification of FLAG surface expression as a function of total channel (HA) expression was analyzed using National Institutes of Health ImageJ. 1.42q as described (14, 18, 19). Data were then normalized to the corresponding control group (100%).

[^3H]Palmitic Acid Incorporation—HEK293 cells were transiently transfected in 6-well cluster dishes ($\sim 3 \times 10^6$ cells per well) with full-length channel constructs as described previously (14). Forty-eight hours after transfection, cells were washed, and 1 ml of fresh DMEM containing 10 mg/ml fatty acid-free BSA was added for 30 min at 37 °C. DMEM/BSA-containing 0.5 mCi/ml [^3H]palmitic acid was added for 4 h at 37 °C. Cells were washed, lysed, and channel fusion proteins were captured by using magnetic microbeads coupled to the HA antibody (μMACS epitope tag isolation kits, Miltenyi Biotec). Proteins were then eluted in SDS/PAGE sample buffer (50 mM Tris-Cl (pH 6.8), 5 mM DTT, 1% SDS, 1 mM EDTA, 0.005% bromophenol blue, 10% glycerol) prewarmed to 95 °C. The recovered samples were separated by SDS/PAGE, transferred to nitrocellulose membranes, and probed with a polyclonal HA antibody (1:1000; Zymed Laboratories, Inc.). A duplicate membrane was exposed to light-sensitive film at -80 °C by using a Kodak Biomax transcreen LE (Amersham Biosciences).

Electrophysiology—Single channel recordings and macro-patch recordings were performed in the excised inside-out configuration of the patch-clamp technique at room temperature. The pipette solution (extracellular) contained 140 mM KCl, 5 mM NaCl, 0.1 μM CaCl_2 , 1 mM MgCl_2 , 20 mM glucose, and 10 mM Hepes (pH 7.3). The bath solution (intracellular) contained 140 mM KCl, 5 mM NaCl, 1 mM MgCl_2 , 20 mM glucose, 10 mM Hepes, (pH 7.3) with intracellular free calcium buffered using 1 mM BAPTA (1,2-bis(o-aminophenoxy)ethane- N,N,N',N' -tetraacetic acid) or dibromo-BAPTA over a range of < 10 nM to 10 μM [Ca^{2+}]. Channel activity was determined during 100-ms step depolarizations over a voltage range from -120 mV to $+120$ mV in 20-mV increments from a holding potential of -80 mV. Tail currents were then examined and normalized to the peak current (100%) in 1 or 10 μM Ca^{2+} and plotted as G/G_{MAX} corresponding to channel activity. Voltage sensitivity was determined by a logarithmic transformation linearizing the activating component of the normalized (G/G_{MAX}) curves in 0.33 μM Ca^{2+} . Data acquisition and voltage protocols were controlled by an Axopatch 200B amplifier and pCLAMP9 software (Axon Instruments). All recordings were sampled at 10 kHz and filtered at 2 kHz. Channel activity was allowed to stabilize for at least 10 min after patch excision before recording.

Basic Hydrophobicity Membrane Interaction Prediction—A basic hydrophobicity (BH) scale algorithm (20) was used to identify potential membrane binding sites in the C terminus of the BK channel by entering the entire C-terminal sequence with respective mutant channels and selecting a window size of 19. A threshold value of 0.6 was identified as the optimal parameter for identifying lipid-binding sites in proteins.

Clustering and Scoring Strategy-palm Prediction—We used the published CSS-palm palmitoylation algorithm (21, 22), to

Electrostatic Switch Controls BK Channel Palmitoylation

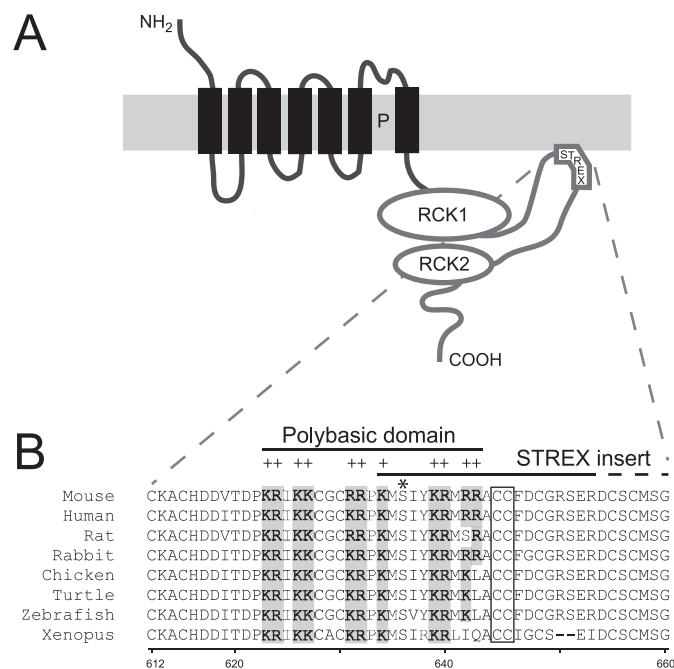


FIGURE 1. Insertion of alternatively spliced STREX insert generates a putative polybasic domain. *A*, schematic illustrating the topology of the BK channel pore-forming α subunit. Inclusion of the alternatively spliced STREX insert at site of splicing C2 generates a putative polybasic domain within the intracellular RCK1-RCK2 linker region of the channel C terminus. *B*, the polybasic domain includes a cluster of basic residues (+) that are evolutionary conserved in vertebrates (basic charge conservation highlighted by gray box). Highlighted within the STREX insert are the conserved palmitoylated cysteine residues, C645:C646 (box outline) and the PKA phosphorylation site, Ser-636 (asterisk). Amino acid numbering is based on the murine channel sequence (accession number AF156674) numbered from the initiating methionine at the MDAL1 start site.

predict cysteine residues within the C-terminal coding sequence of the murine BK channel. Sequences were analyzed with CSS-palm v2.0.4. The palmitoylation prediction threshold was set to the highest cutoff.

Statistics—All statistical analyses were performed using GraphPad Prism or Igor Pro using one-way analysis of variance with Tukey and/or Newman-Keuls post hoc tests for significance between groups.

RESULTS

A Putative Membrane Targeting Polybasic Domain Upstream of the Palmitoylated C-terminal Cysteines—Incorporation of the STREX splice insert at the C2 site of alternative splicing (Fig. 1*A*) generates a cluster of 11 basic amino acid residues of 21 (Lys-623 to Arg-R643) that may form a polybasic domain. Sequence alignment of these positively charged amino acids just upstream of the previously identified C-terminal STREX palmitoylation site (C645:C646) (14) shows evolutionary conservation across the vertebrate phyla, suggesting that these basic residues may be of functional significance (Fig. 1*B*).

Using a BH algorithm that can identify potential membrane binding sites in less structured regions of proteins (20), the potential of the polybasic domain to function as a membrane-targeting domain was determined. Examination of the intracellular STREX C terminus (S6:STREX) sequence identified the polybasic domain as a potential membrane interaction site, with maximum values recorded between residues 633–635 of

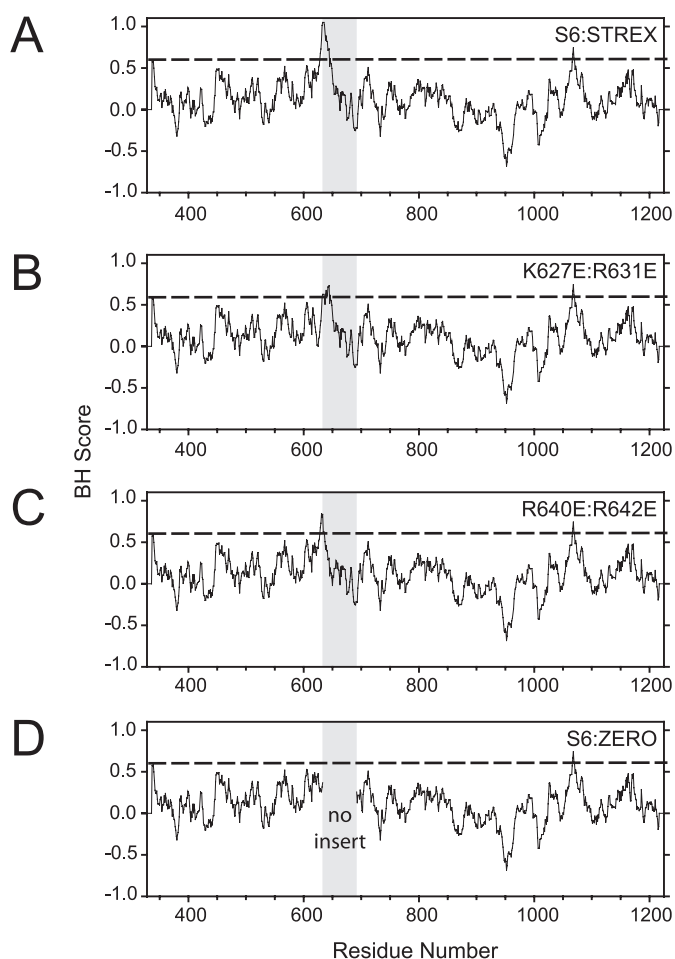


FIGURE 2. BH profiles of the STREX C terminus with mutations in the putative polybasic domain. *A*, the C terminus of the BK channel containing the STREX insert (gray box) contains a predicted membrane binding site in the identified polybasic domain (peak residues 633–635). Mutation of basic to acidic residues in the region distal to STREX (K627E:R631E) (*B*) and the region proximal to the cysteine palmitoylation site in STREX (R640E:R642E) (*C*). *D*, the ZERO “insertless” splice variant of the C terminus lacking STREX predicts no major membrane binding sites. The shaded box indicates location of the alternatively spliced STREX insert.

1.05 (Fig. 2*A*). To define basic residues that would disrupt the membrane-targeting function of the polybasic domain identified by the BH algorithm, point mutations of basic residues in two distinct regions of the polybasic domain were identified.

Mutation of two basic residues to negatively charged acidic residues either in the distal basic region immediately upstream of the STREX insert, Lys-627 and Arg-631, or in the proximal basic region within the STREX insert, Arg-640 and Arg-642, decreased the BH probability score for the peak plasma membrane interaction site to 0.63 (K627E:R631E) and 0.84 (R640E:R642E) (Fig. 2, *B* and *C*). Mutation with neutral (alanine) residues reduced but did not abolish the membrane interaction prediction. BH scores were 0.82 (K627A:R631A) and 0.91 (R640A:R642A). Furthermore, deletion of the downstream basic residues in the STREX insert (by using the insertless ZERO channel C terminus sequence (S6:ZERO) in which the STREX insert is excluded) also abolished the predicted membrane interaction score, suggesting that the upstream region (six basic residues) alone does not function as a polybasic domain (Fig. 2*D*). Thus, basic residues immediately upstream

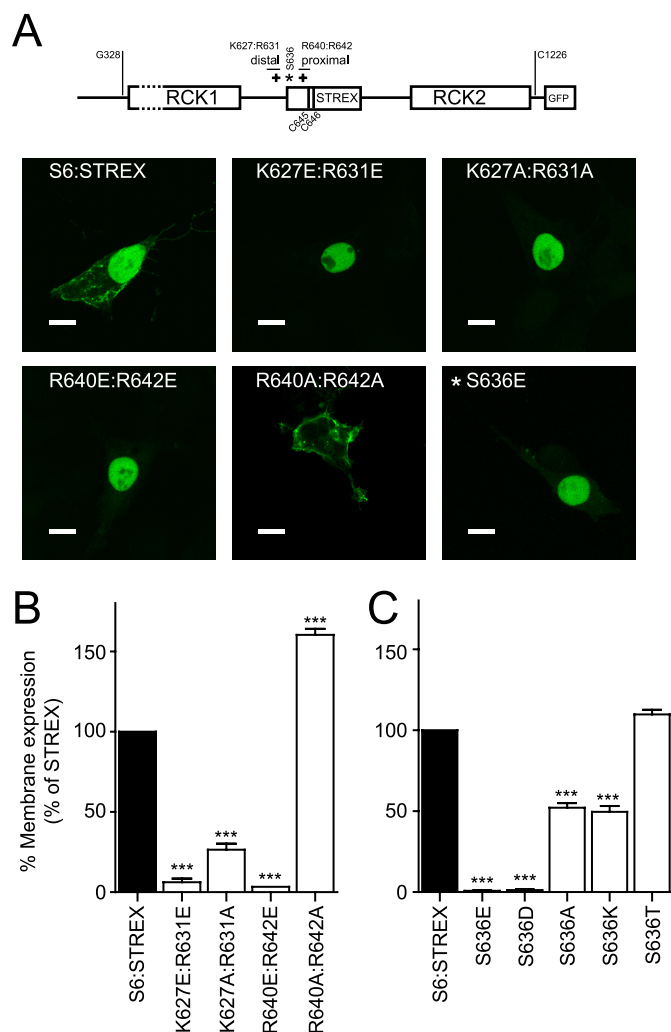


FIGURE 3. The polybasic domain regulates plasma membrane localization of the STREX C terminus. A, schematic of the STREX C-terminal-GFP fusion construct is illustrated. Distal and proximal polybasic sites are indicated relative to the C645:C646 palmitoylation site, and the Ser-636 PKA phosphorylation site is indicated by an asterisk. Shown are representative single cell confocal images of C-terminal STREX (S6:STREX) and the corresponding C-terminal STREX polybasic mutants K627E:R631E, K627A:R631A, R640E:R642E, and R640A:R642A and the phosphomimetic mutant S636E, expressed in HEK293 cells. Scale bars = 10 μ m. B, summary bar graph illustrating the effect of the polybasic mutations on STREX C-terminal construct localization at the plasma membrane expressed as a percentage of S6:STREX plasma membrane expression (where S6:STREX is 100%). C, the effect of phosphomimetic (E and D), neutral (A), basic (K), and conservative (T) mutations of the Ser-636 PKA phosphorylation site on STREX C-terminal construct localization at the plasma membrane. All data are mean \pm S.E. $n > 6$, $n > 490$. ***, $p < 0.001$ compared with S6:STREX (analysis of variance with Tukey post hoc test).

(distal) and within STREX (proximal) are required to generate a putative polybasic domain.

Mutation of the previously identified palmitoylated cysteines within STREX, which are essential for plasma membrane association of the STREX C-terminal domain (14), to alanine (C645A:C646A) did not affect the BH probability score (1.05).

Disruption of the Polybasic Domain Decreases Association of the STREX C Terminus at the Plasma Membrane—To determine whether disruption of the polybasic domain affects membrane association of the STREX C terminus, fluorescently labeled C-terminal constructs were generated as described pre-

viously (14). Expression of the STREX C terminus (S6:STREX) in HEK293 cells resulted in robust localization of the GFP fusion construct at the plasma membrane (Fig. 3, A and B). Site-directed mutagenesis was undertaken to disrupt the basic charge within the polybasic domain by substituting basic residues with negatively charged (glutamic acid) amino acids and to neutralize the basic charge by substituting with neutrally charged (alanine) amino acids. In the distal region of the polybasic domain, just upstream of the STREX insert, substitution with negative (glutamic acid) residues (K627E:R631E) or neutral (alanine) residues (K627A:R631A) decreased membrane targeting to 6.3% and 26.5% of the wild-type S6:STREX membrane expression, respectively (Fig. 3, A and B), with the fluorescent fusion proteins now located predominantly within the nucleus. Mutation of the basic region proximal to the previously identified palmitoylation site in STREX with negative (glutamic acid) residues (R640E:R642E) also abolished membrane targeting to 0.8%, with targeting again redirected to the nucleus. However, mutation of these proximal basic residues to neutral (alanine) residues (R640A:R642A) demonstrated that fluorescence at the plasma membrane actually increased to 160% of the control S6:STREX value (Fig. 3, A and B). Nuclear localization was also substantially decreased in the (R640A:R642A) mutant, with only 11.5% of cells also showing fluorescence in the nucleus. Therefore, distinct regions of the polybasic domain appear to play an important role in controlling localization of the STREX C terminus at the plasma membrane.

The Polybasic Domain Regulates the Palmitoylation Status of the STREX Insert—Mutation of the two palmitoylated cysteine residues to alanine (C645A:C646A) essentially abolishes plasma membrane association of the S6:STREX construct (14) suggesting that the polybasic domain *per se* is not sufficient for stable plasma membrane association. Thus, to address whether inhibition of membrane association of the STREX C terminus upon disruption of the polybasic domain results from a change in palmitoylation status of the STREX domain, we examined radiolabeled [3 H]palmitate incorporation in the polybasic mutant constructs expressed in HEK293 cells (Fig. 4A). STREX C terminus (S6:STREX) was robustly palmitoylated by endogenous acyl palmitoyltransferases. Disruption of the polybasic domain by substituting negatively charged (glutamic acid) residues in both identified regions of the polybasic domain (K627E:R631E and R640E:R642E) effectively abolished [3 H]palmitate incorporation. Neutral (alanine) substitution of the region distal to STREX (K627A:R631A) also substantially reduced [3 H]palmitate incorporation to \sim 30% of S6:STREX. These data suggest that the polybasic domain must play an important role in regulating the palmitoylation status of the C645A:C646A palmitoylation motif. Interestingly, the R640A:R642A STREX C-terminal mutant demonstrated an increased level of palmitoylation to \sim 390% of S6:STREX. Therefore, neutral substitution of basic residues in the region closest to the identified palmitoylation motif within STREX can increase palmitoylation of the C-terminal constructs in agreement with the imaging experiments (Fig. 3, A and B). This suggests that these different polybasic mutations may be reporting differences in local structure/folding required for efficient palmitoylation and membrane trafficking of the S6:STREX C-terminal

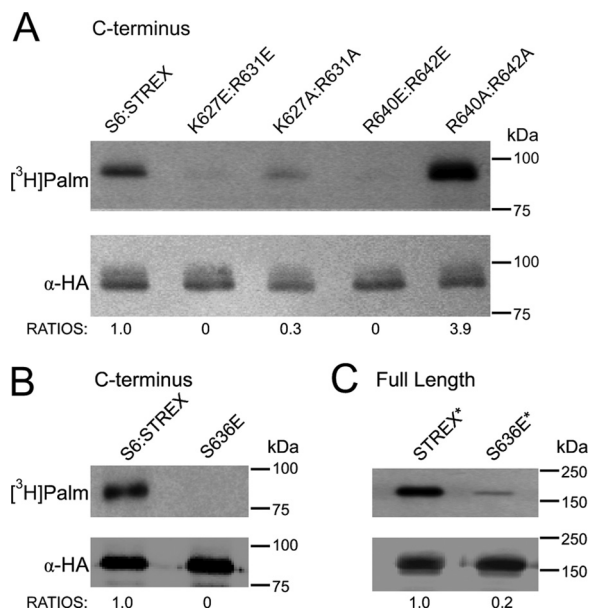


FIGURE 4. Palmitoylation of STREX is modulated by the polybasic domain. A, representative fluorographs (upper panel) and Western blot analyses (lower panel) of HA-tagged C-terminal S6:STREX and the corresponding polybasic mutant C-terminal constructs K627E:R631E, K627A:R631A, R640E:R642E, and R640A:R642A or (B) the C-terminal phosphomimetic construct S636E. C, full-length channel STREX channels (STREX*) were examined, in which the constitutively expressed palmitoylated cysteine residues site in the S0-S1 loop were mutated to alanine (C53A:C54A:C56A) to allow examination of changes in STREX domain palmitoylation *per se* in full-length channels. STREX* and the corresponding full-length phosphomimetic S636E* construct were examined. All constructs were expressed in HEK293 cells and labeled with [³H]palmitate for 4 h and immunoprecipitated (IP) by using α-HA magnetic microbeads and detected by fluorography. Ratios indicate palmitoylation of respective construct normalized to protein expression and expressed as a fraction of the corresponding S6:STREX or STREX* control (1.0).

construct. Robust expression in Western blot analysis demonstrated that the change in palmitoylation status of the channel constructs was not the result of altered synthesis or degradation of the C terminus channel fusion proteins (Fig. 4A).

PKA Phosphorylation as an Electrostatic Switch to Control Channel Palmitoylation?—We showed previously that phosphorylation of a conserved serine residue (Ser-636, Figs. 1B and 3A) results in dissociation of the STREX domain from the plasma membrane, leading to channel inhibition (14). The Ser-636 residue is a PKA consensus site located close to the midpoint of the polybasic domain and immediately upstream of the cysteine palmitoylation motif (C645:C646). As phosphorylation of Ser-636 would introduce a negative charge into the otherwise polybasic region, we hypothesized that phosphorylation of Ser-636 may act as an electrostatic switch to control palmitoylation of the STREX domain and its association with the plasma membrane. To test this, we generated a phosphomimetic mutation with a negatively charged glutamic acid residue at the Ser-636 site. This phosphomimetic mutation, S636E, slightly reduced the BH probability score for the domain to 0.95, suggesting that phosphorylation of Ser-636 may disrupt the functional polybasic domain. Phosphomimetic substitution essentially abolished C-terminal localization of the STREX C terminus at the plasma membrane (Fig. 3, A and C), with membrane expression of S636E at $0.8 \pm 0.3\%$ of the wild-type STREX C terminus (C). A negatively charged aspartic acid res-

idue had the same effect, abolishing STREX C-terminal localization at the plasma membrane ($1.3 \pm 0.4\%$ of the wild-type STREX C terminus). To establish whether this is a charge-specific effect, as would be predicted if phosphorylation of Ser-636 was acting as an electrostatic switch upon introducing a negative charge, we also mutated Ser-636 to a neutral alanine or positively charged lysine residue. Although both the alanine and lysine mutations reduced membrane expression of the STREX C terminus, this was only by $52 \pm 2.9\%$ and $49.5 \pm 3.6\%$, respectively, compared with the abolition of membrane expression with the phosphomimetic glutamic acid and aspartic acid mutants. This supports a model in which phosphorylation of Ser-636 acts as an electrostatic switch. However, we cannot exclude that mutation of Ser-636 *per se* does not also result in changes in local structure/folding. In support of this, conservative mutation of Ser-636 to threonine had no effect on STREX C terminus membrane expression. However, as both serine and threonine could be phosphorylated by PKA *in vivo*, an alternative explanation could be that cycles of phosphorylation/dephosphorylation are also important for trafficking of the isolated STREX C terminus to the plasma membrane.

To determine whether phosphorylation of Ser-636 could control palmitoylation of the C645:C646 site, we examined palmitoylation in both the STREX C terminus and full-length channel constructs. The S636E phosphomimetic mutation significantly reduced [³H]palmitate incorporation into STREX C-terminal constructs (Fig. 4B) without affecting protein expression, suggesting that Ser-636 phosphorylation is important for controlling palmitoylation of STREX. We also examined the effect of the S636E phosphomimetic mutation in full-length STREX channels in which the constitutively expressed palmitoylated cysteine residues located in the S0-S1 linker (15) were mutated to alanine residues (C53A:C54A:C56A). This allowed the effect of the S636E mutation on palmitoylation of the STREX domain alone to be analyzed in the intact channel. In the full-length STREX channel with the S0-S1 palmitoylation sites mutated to alanine (STREX*), palmitoylation was robust, demonstrating efficient palmitoylation of the STREX insert. However, channels with the S636E phosphomimetic mutation (S636E*) displayed a significantly reduced [³H]palmitate incorporation to $\sim 20\%$ of the STREX* channel (Fig. 4C). Taken together, these data support a model in which the polybasic domain is a phosphorylation-dependent electrostatic switch that controls palmitoylation of the STREX domain of the BK channel.

Disruption of the Polybasic Domain Affects Trafficking and Biophysical Properties of the BK Channel—The evidence so far demonstrates that the polybasic region controls palmitoylation of the STREX domain. We next examined the functional impact of mutating the polybasic domain in the full-length STREX splice variant of the BK channel. We have shown that palmitoylation controls membrane expression of the STREX C terminus, therefore we asked whether membrane expression of full-length channels could be controlled by STREX palmitoylation. To do this we exploited a quantitative immunofluorescence assay of cell surface expression whereby channels expressed at the cell surface were quantified by probing for a FLAG epitope at the extracellular N terminus of the channel in

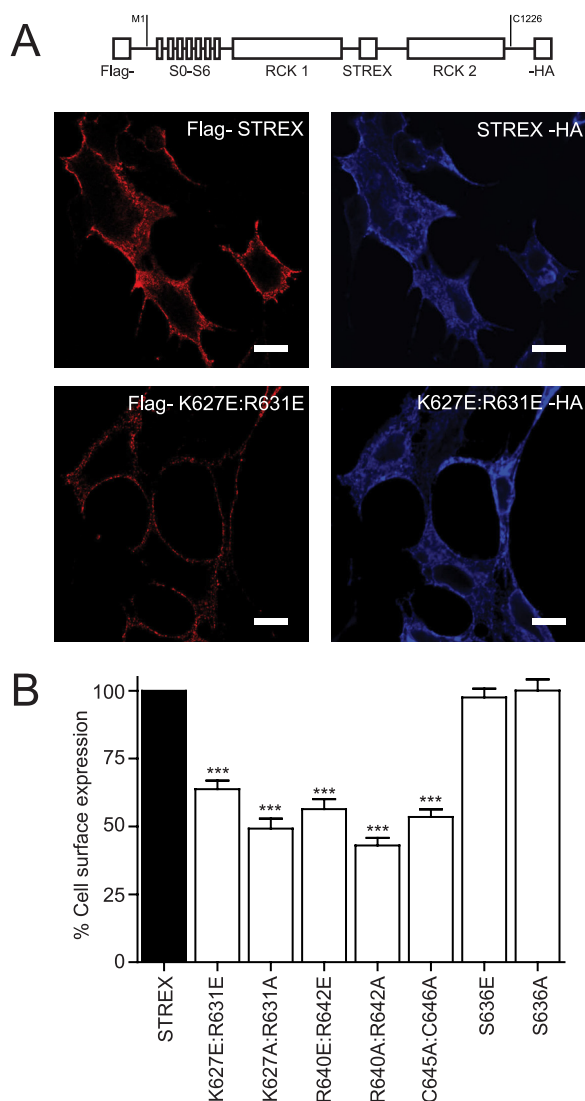


FIGURE 5. The polybasic domain also regulates cell surface expression of the full-length STREX channel. *A*, schematic of the N-terminal FLAG-tagged and C-terminal HA-tagged full-length STREX variant of the BK channel with the transmembrane domains (S0-S6). Representative confocal images of wild-type STREX and the K627E:R631E polybasic mutants are shown expressed in HEK293 cells. Scale bars = 10 μ m. *B*, summary bar graph of cell surface membrane expression of STREX, the various polybasic mutant channels, phosphomimetic mutations at Ser-636, and the STREX palmitoylation mutant channel C645A:C646A. Surface expression for each site-directed mutant is normalized to STREX plasma membrane expression (STREX = 100%). All data are means \pm S.E. $n = > 3$, $n > 50$. ***, $p < 0.001$ compared with STREX (analysis of variance with Tukey post hoc test).

non-permeabilized conditions (Fig. 5A). Total channel expression was then assayed under permeabilized conditions and probed for an HA epitope on the intracellular C terminus of the channel, as described previously (15, 19).

The K627E:R631E, K627A:R631A, R640E:R642E and C645A:C646A mutants all displayed a significant decrease (by $\sim 50\%$) in expression at the plasma membrane, as might be predicted from the largely abolished palmitoylation in the STREX C terminus mutants (Fig. 5B). However, the R640A:R642A polybasic mutant, which displays an increase in palmitoylation and increased expression of the STREX C terminus construct at the plasma membrane, also showed a similar reduction in cell surface expression. Furthermore, the S636E mutant, which shows

a decrease in STREX palmitoylation, had normal cell surface expression similar to the wild-type, full-length STREX channel. The S636A mutant also had normal cell surface expression. Thus, there was no correlation between palmitoylation status of STREX and the cell surface expression of full-length channels as observed with the isolated STREX C terminus. This suggests that a change in palmitoylation, upon disruption of the polybasic domain, is not the only determinant of cell surface expression. In support of this, the region upstream of the polybasic domain includes multiple acidic trafficking signals that are important for cell surface expression (19). Thus, mutations within the polybasic domain may modulate cell surface trafficking by affecting these acidic trafficking motifs independently of changes in palmitoylation status.

We next examined whether the biophysical properties of the polybasic mutant channels were modified using patch-clamp electrophysiology. Channel constructs were transiently transfected into HEK293 cells and analyzed in inside-out patches using symmetrical (140 mM) potassium gradients.

Polybasic domain mutations in full-length STREX channels had no significant effect on single channel conductance, with STREX displaying a mean slope conductance of 244 ± 4.4 pS under the recording conditions used (Fig. 6, A and B). To investigate whether disruption of the polybasic domain affects channel activity, macroscopic BK currents were assayed over a voltage range of -120 mV to $+120$ mV under different intracellular free calcium conditions (Fig. 7). In the absence of intracellular free calcium ($[Ca^{2+}]_i$ buffered to < 10 nM) no significant differences in the half maximal voltage for activation ($V_{0.5_{MAX}}$) were observed between STREX channels or the polybasic mutants, suggesting that the intrinsic voltage dependence of the channel is not modified.

However, at low (0.33μ M) micromolar $[Ca^{2+}]_i$, the concentration range over which the apparent calcium sensitivity of the STREX variant of the channel shows the largest difference to the insertless ZERO variant (18, 23, 24), polybasic mutant channels displayed significant differences in $V_{0.5_{MAX}}$. The $V_{0.5_{MAX}}$ of the STREX channel in 0.33μ M $[Ca^{2+}]_i$ was 40.6 ± 2.5 mV. Under identical conditions, the insertless ZERO channel shows a significant rightward shift in the half maximal voltage for activation of 77.6 ± 3.5 mV (Fig. 7C), as reported previously (18, 23, 24). The negative charges substituted within the polybasic domain distal to STREX, K627E:R631E, resulted in a significant ($\sim +31$ mV) rightward shift in the $V_{0.5_{MAX}}$ to 71.9 ± 7.8 mV. Disruption of the basic region proximal to the cysteine palmitoylation site in STREX, also with negative residues, R640E:R642E, also significantly right-shifted the $V_{0.5_{MAX}}$ to 59.7 ± 4.7 mV (Fig. 7, B and C). This rightward shift characterizes a shift toward the ZERO channel phenotype and is a similar shift to that observed for the palmitoylation-deficient mutant channel (C645A:C646A), which displays a $V_{0.5_{MAX}}$ of 65.5 ± 8.1 mV under the same conditions (Fig. 7C), as well as STREX channels that are inhibited by PKA-dependent phosphorylation (14).

Mutation with neutral (alanine) residues in the polybasic region distal to STREX, K627A:R631A, had no significant effect on $V_{0.5_{MAX}}$ (54.3 ± 10.5 mV) (Fig. 7C). However, mutation of the basic residues within STREX to alanine, R640A:R642A,

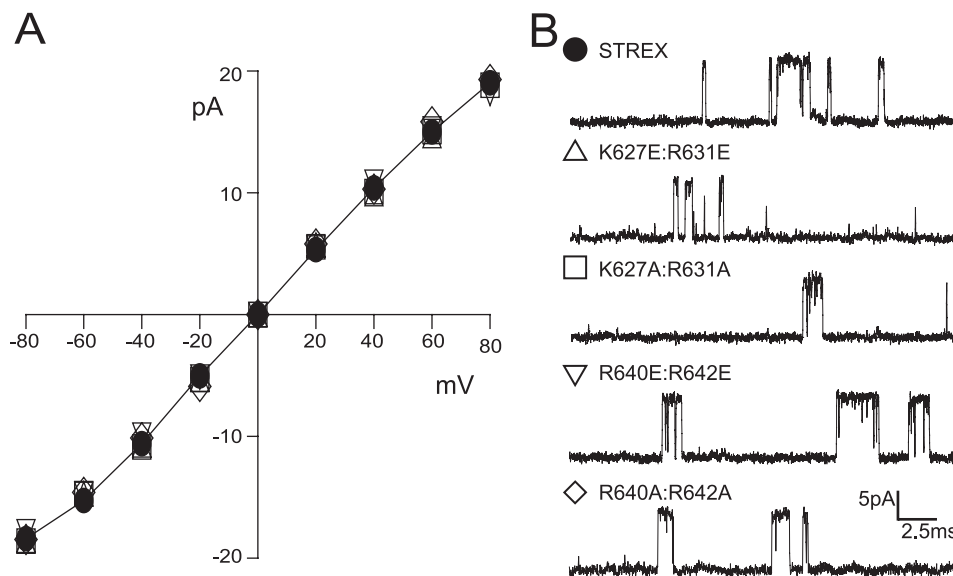


FIGURE 6. Single channel amplitude is not affected by disruption of polybasic domain. *A*, unitary current voltage plot for wild-type STREX channels (closed circles) and the indicated polybasic domain site directed mutants K627E:R631E (upright triangles), K627A:R631A (squares), R640E:R642E (inverted triangles) and R640A:R642A (diamonds), ($n = 3$). Recordings were made in excised inside-out patches in symmetrical 140 mM K^+ gradients with 0.33 μM intracellular free Ca^{2+} . *B*, representative single channel recordings from excised inside-out patches recorded as in *A* at +40 mV.

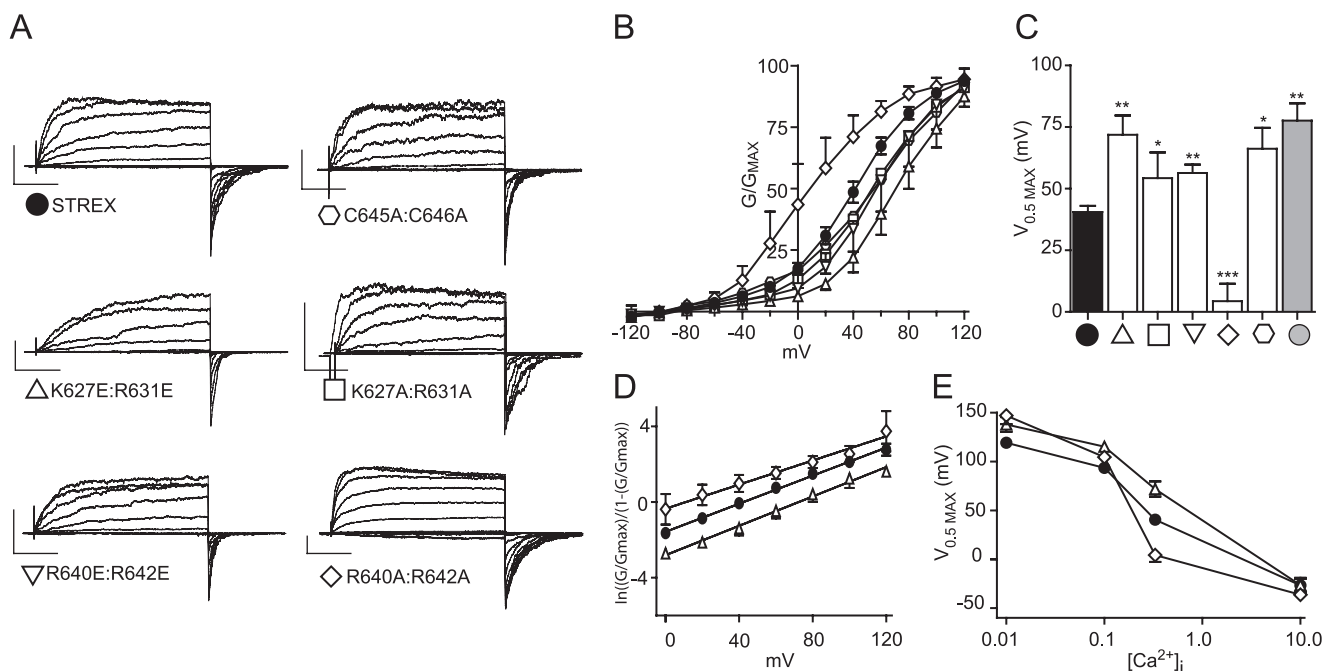


FIGURE 7. Effect of palmitoylation and polybasic mutations on STREX channel voltage for half-maximal activation. *A*, representative macropatch recordings showing BK currents in response to a depolarizing voltage step protocol (-120 mV to +120 mV) from a holding potential of -80 mV. All recordings were from excised inside-out patch recordings in symmetrical 140 mM K^+ gradients and with intracellular free calcium buffered using 1 mM BAPTA or dibromo-BAPTA over a range of < 10 nM to 10 μM $[Ca^{2+}]_i$ in HEK293 cells. STREX (closed circles), K627E:R631E (upright triangles), K627A:R631A (squares), R640E:R642E (inverted triangles), R640A:R642A (diamonds) and palmitoylation-deficient mutant C645A:C646A (hexagon). Scale bars = 1 nA/25 ms. *B*, normalized G/G_{MAX} conductance curves of STREX and the respective polybasic mutant channels. *C*, summary bar graph showing $V_{0.5 MAX}$ of the STREX channel and the polybasic mutant channels, STREX palmitoylation mutant channel, C645A:C646A (hexagon) and insertless ZERO channel variant (gray circles). *D*, logarithmic transformation of activation curves in *B*. *E*, voltages of half-maximal channel activations ($V_{0.5 MAX}$), at different $[Ca^{2+}]_i$, are shown for STREX (closed circles), K627E:R631E (upright triangles), and R640A:R642A (diamonds) determined from single Boltzmann fits as in *B*. All data are means \pm S.E. ($n = 4-8$). **, $p < 0.01$, compared with STREX (analysis of variance with Neumann Keuls post hoc test).

resulted in a significant leftward shift (~ -35 mV) compared with STREX, with a $V_{0.5 MAX}$ of 4.4 ± 7.1 mV (Fig. 7C). This observation is in line with the observed increase in STREX palmitoylation and domain association with the plasma membrane (Figs. 3B and 4A). Voltage sensitivity of the polybasic mutant channels at 0.33 μM $[Ca^{2+}]_i$ was not different to STREX

(Fig. 7D), as assessed by logarithmic transformation of the activating component of the normalized (G/G_{MAX}) curves against depolarized potentials (0 mV to 120 mV), as shown in Fig. 7B. This also supports the observations seen in the absence (<10 nM) of intracellular free calcium. However, at high (10 μM) micromolar $[Ca^{2+}]_i$, the $V_{0.5 MAX}$ values converged so that

there was again no significant difference in $V_{0.5_{MAX}}$ between STREX and the polybasic domain variants. At high micromolar calcium, the $V_{0.5_{MAX}}$ values for STREX and the insertless ZERO variant also converged suggesting that the effect of palmitoylation and the polybasic domain are primarily exerted at low micromolar concentrations of $[Ca^{2+}]_i$, the range over which STREX and ZERO properties are most divergent.

DISCUSSION

We have identified a series of basic residues immediately upstream of the palmitoylated cysteine residues within the intracellular C terminus of the STREX BK channel that are important for control of STREX domain palmitoylation and channel properties. Importantly, a PKA phosphorylation site (Ser-636) within this polybasic domain that controls STREX domain association with the plasma membrane (14) controls STREX insert palmitoylation. As phosphomimetic, but not neutral or basic amino acid substitutions of Ser-636, abolished STREX membrane association, our data support a model in which phosphorylation acts as an electrostatic switch to control the polybasic domain.

A Polybasic Domain Controls Palmitoylation of the BK Channel C Terminus—The predicted polybasic region in the BK channel C terminus comprises 11 basic residues in a stretch of 21 amino acids. The polybasic region is highly conserved across vertebrates, as are the sites for palmitoylation and phosphorylation within the STREX insert.

Mutation of basic amino acids to acidic residues within either the distal (K627E:R631E) or proximal (R640E:R642E) region of the polybasic domain resulted in significant reduction in the ability of the isolated STREX C terminus of the channel to associate with the plasma membrane. This reduction in cell surface expression was associated with abrogation of palmitoylation of the STREX insert. The CSS-palm cysteine palmitoylation algorithm (21, 22) also predicts that acidic mutation of either the distal or proximal basic residues reduces the palmitoylation score for the double cysteine motif that is palmitoylated in STREX (data not shown).

The functional effect of introducing a negative charge into the polybasic domain was to shift the $V_{0.5_{MAX}}$ of the STREX channel rightwards at low ($0.33 \mu M [Ca^{2+}]_i$) toward that observed in channels in which the palmitoylated cysteines are mutated to alanine or in which the STREX insert is excluded (ZERO variant). Polybasic mutations had no significant effect on the voltage dependence of the channel, as there was no change in the $V_{0.5_{MAX}}$ in nominally calcium-free ($<10 \text{ nM}$) conditions or in the voltage-sensitive component of the mutants at $0.33 \mu M [Ca^{2+}]_i$. Furthermore, at high micromolar $[Ca^{2+}]_i$ ($10 \mu M$), the difference in $V_{0.5_{MAX}}$ was lost between variants. Thus, although the polybasic region is important for controlling STREX insert palmitoylation, the functional effect on channel activation is only manifest at low micromolar $[Ca^{2+}]_i$ concentrations. Functionally, this may be important, as this is the range of $[Ca^{2+}]_i$ over which STREX variant channels show the largest differences between other variants and levels routinely achieved in excitable cells.

Mutation of basic residues to neutral alanine residues showed very significant position effects. Neutralization of the

basic region more distal (K627A:R631A) to the palmitoylated cysteine residues also reduced STREX palmitoylation and resulted in a right shift in $V_{0.5_{MAX}}$. In contrast, charge neutralization of the basic residues (R640A:R642A) closest to the palmitoylated cysteines dramatically increased STREX palmitoylation. Enhanced plasma membrane targeting of the isolated STREX C terminus promoted a significant leftward shift in $V_{0.5_{MAX}}$. Mutation of R640A:R642A was also predicted by the CSS-palm palmitoylation prediction algorithm to increase the palmitoylation prediction score (data not shown). Taken together, this suggests that basic residues within the distal and proximal regions of the polybasic region play important but also distinct functions in controlling palmitoylation of the STREX insert.

The effects of neutral or acidic mutations in the polybasic domain on channel $V_{0.5_{MAX}}$ paralleled the changes observed in palmitoylation of the STREX insert, suggesting that palmitoylation is a major determinant controlling channel gating at low micromolar calcium concentrations. In addition, the polybasic mutations also had significant effects on cell surface expression of the STREX channel. Polybasic mutations that decreased STREX palmitoylation reduced cell surface expression of the full-length channel to a similar extent as that observed with the palmitoylation-deficient mutant C645A:C646A. However, as the R640A:R642A mutant that displays increased palmitoylation compared with STREX also displayed reduced surface expression and the fact that the S636E mutant that reduces palmitoylation had no significant effect on cell surface expression of full-length channels, suggests that additional mechanisms, rather than palmitoylation of the STREX insert alone, are responsible for controlling cell surface expression. The region immediately upstream of the polybasic domain includes multiple acidic trafficking motifs (19). Therefore, mutations within the polybasic domain may affect cell surface trafficking through effects on these acidic trafficking motifs as well as through changes in palmitoylation of the STREX insert.

The contribution of surrounding residues in controlling palmitoylation of target cysteines is very poorly understood. In some proteins, sites of palmitoylation are close to hydrophobic domains such as transmembrane domains or sites of alternative, irreversible lipid modifications such as myristoylation (8, 9). Palmitoylation of cysteines is dependent on a number of factors, including localization with the membrane acyl palmitoyltransferases (zDHHCs) (1, 7) and the local concentration of fatty acid CoA, in part determined by local hydrophobic environments (11). Thus, if the polybasic domain acts as a potential (initiating) membrane association domain, it would place the STREX domain in an environment accessible to zDHHCs with appropriate fatty acid CoA ester concentrations. On the basis of our data, such a function would likely require the entire polybasic domain.

In some proteins, basic residues vicinal to the palmitoylated cysteines have also been reported to promote cysteine reactivity and sensitivity to nucleophilic attack to increase the efficiency of cysteine palmitoylation (11, 12, 25). However, although acidic mutation of the proximal basic residues decreased palmitoylation, mutation of these basic residues to alanine, in fact, resulted in enhanced STREX palmitoylation, leading to a gain

of function of the STREX channel. This was not observed upon alanine mutation of the distal basic residues. This suggests that the specific environment surrounding the target cysteine residues in STREX may also be critical for efficient palmitoylation, not just the basic residues *per se*, as demonstrated in other proteins (for example, Refs. 26–28). Whether the increased palmitoylation of the STREX domain results from an increased efficiency of palmitoylation of endogenously palmitoylated cysteines in this system (C645:C646) or allows other potential cysteines with STREX, such as Cys-649 (29), to be palmitoylated by endogenous zDHHCs remains to be determined.

The polybasic region and alternatively spliced STREX insert lie within the predicted unstructured linker (30) between the two regulator of potassium conductance (RCK) domains of the intracellular C terminus of the BK channel. On the basis of the crystal structure of the RCK domains, the linker is predicted to be positioned peripherally to the main RCK1-RCK2 gating domain (31–33). This would place the polybasic linker in a location accessible to the plasma membrane. The basic domains of some proteins bind either nonspecifically to monovalent acidic phospholipids (for example Src, K-Ras, and myristoylated alanine-rich C-kinase substrate (34, 35)) or laterally sequester phosphoinositides such as PI(4,5)P₂ in the plasma membrane (such as small GTPases (10, 34)). Polybasic domains of proteins that interact electrostatically with the plasma membrane, in contrast to interactions with DNA in the nucleus, typically display a “flat” rather than curved structure (35, 36). Therefore, whether the polybasic domain constitutes a linear structure electrostatically attracted to the negatively charged lipid membrane and with an anchoring palmitoylation site that penetrates the lipid bilayer at the C-terminal end remains to be determined.

However, although the BH prediction algorithm (20) suggested that the polybasic domain may serve as an independent membrane-targeting domain, the polybasic region alone is not sufficient to support robust membrane expression of the STREX C terminus in the absence of palmitoylation of the STREX insert (14). Thus, the polybasic domain may control STREX palmitoylation independently of acting as a membrane targeting domain by controlling local cysteine reactivity.

Phosphorylation of the Polybasic Domain as an Electrostatic Switch to Control Palmitoylation?—Palmitoylation is the only reversible lipid modification because of the action of palmitoyltransferases and acyl thioesterases. The identification of the polybasic region of the BK channel as an important determinant of STREX palmitoylation also suggests a mechanism to allow cross-talk between protein phosphorylation and protein palmitoylation.

Within the STREX domain, an evolutionary conserved serine residue, Ser-636, located centrally in the polybasic domain, is phosphorylated by cAMP-dependent protein kinase (PKA) and is important for inhibition of STREX channel activity (16, 17). The polybasic domain includes six arginine residues and five lysine residues with no acidic residues and, therefore, would be predicted to have a charge of about +11 at a neutral pH (37, 38). Phosphorylation of a serine residue within a protein introduces a phosphoryl group that adds two negative charges (37). Thus, phosphorylation at the STREX Ser-636

PKA phosphorylation site would be expected to switch the overall net charge of the region to +9. Crucially, it would theoretically break the large polybasic domain into two smaller basic regions with a net charge of between +5 and +6 and +2 and +3. These smaller basic regions on their own perhaps are insufficient to facilitate robust palmitoylation of the STREX domain in accordance with the mutagenic analysis of the basic residues at K627:R631 and R640:R642.

Although introducing neutral (arginine) or positive (lysine) charge at Ser-636 also reduced association of the STREX domain with the plasma membrane, the inhibitory effect was significantly smaller than that observed by introducing phosphomimetic (negative charge with glutamic acid or aspartic acid) mutations that abolished membrane expression of STREX. The conservative mutation (to threonine) was without effect. Crucially for the full-length STREX channel (which had been engineered so that palmitoylation could only occur at the STREX site and not at the N-terminal S0-S1 loop), phosphomimetic mutation of the serine residue (S636E) significantly reduced [³H]palmitate incorporation into the STREX domain to compliment the data from the STREX C-terminal constructs. Taken together, our data support a model in which phosphorylation of Ser-636 acts as an electrostatic switch to control STREX function. However, we cannot fully exclude that the effect of phosphorylation, or phosphomimetic mutation, also has an effect on local folding/structure *per se*. Whether phosphorylation reduces the efficacy of STREX palmitoylation by its cognate zDHHCs (29) or may additionally enhance accessibility of acyl thioesterases warrants further examination.

In conclusion, we demonstrate that an evolutionary conserved polybasic region in the C terminus of BK channels is an important determinant of channel palmitoylation. Importantly, phosphorylation of the polybasic region likely acts as an electrostatic switch to control palmitoylation of adjacent cysteine residues. Increasing evidence supports a role for phosphorylation in controlling palmitoylation of other signaling proteins such as the phosphodiesterase 10A (25). The control of protein palmitoylation by a phosphorylation-dependent electrostatic switch is likely to represent an important regulatory mechanism to allow cross-talk between the two major posttranslational mechanisms of protein phosphorylation and protein palmitoylation.

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